ANALYTICAL ISOELECTRIC FOCUSING IN POLYMERIZABLE THIN LAYERS CONTAINING SEPHADEX

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1. Introduction

Isoelectric focusing (IEF) has, in recent years, become an extremely sensitive and widely employed tool for the detection of very small differences in the isoelectric points (pI) of various substances, including proteins and RNAs [1]. For comparison of multiple samples, analytical IEF in thin-layers of polyacrylamide has proved to be of considerable value, since the gel plates are easy to handle, the resolution of components is excellent [2] and the focused substances can be detected by a number of methods [1]. However, very large molecules cannot be focused in these gels either because they do not enter the gel or because excessively long running times are required to reach equilibrium. These drawbacks can be overcome by using Sephadex as anti-convective medium [3]. Focused proteins are identifiable by a paper print which is made of the Sephadex thin-layer after the focusing process. The paper is dried in an oven and then stained [3]. Enzymes can be detected by substrate impregnated papers [4].

We have developed a simpler technique in which substances are focused in a Sephadex layer in the presence of acrylamide monomer and a cross-linking agent. After IEF, the whole layer is polymerized by spraying a solution containing polymerization initiators in a concentrated buffer onto the gel. Focused material can then be identified by standard methods. The advantages of this new approach are the possibility of focusing very large molecules or even viruses on thin-layers combined with the ease of handling a polymerized gel and the use of a variety of established methods to detect the focused substances. The disadvantages of IEF in sucrose gradients (bad resolution or isoelectric precipitation of components, long running times) and in polyacrylamide thin-layers (possible steric hindrance, risk of artifacts due to the presence of polymerization catalysts) are thus avoided; essentially only the desirable sides of both methods are retained.

2. Materials and methods

Tomato Bushy Stunt Virus (TBSV) was grown and purified as described before [5]. As judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, the preparation contained only the two proteins characteristic of a clean TBSV-sample. Lyophilized human Bence-Jones protein Whi was a gift of Dr J. R. L. Pink, Basel. Sperm whale myoglobin was obtained from Calbiochem, USA. Samples of two hyperimmune rabbit antistreptococcal group A variant polysaccharide sera were kindly donated by Dr D. G. Braun, Basel.

Sephadex G-75 superfine was purchased from Pharmacia, Uppsala, Sweden; carrier ampholytes 'Ampholine' of various pH ranges from LKB produkter AB, Stockholm, Sweden; acrylamide (twice crystallized) and *N,N'*-methylene-bisacrylamide from Serva Entwicklungslabor, Heidelberg, Germany; all other chemicals employed were of analytical grade.

15 g Sephadex G-75 superfine was stirred overnight at room temperature in 280 ml of a solution containing 4% (w/v) acrylamide, 0.13% (w/v) *N,N'*-methylene-bisacrylamide and 2.8% (w/v) carrier ampholytes of different pH ranges. Glass plates (21.6 × 16.8 cm, gelatin-coated, Ilford, Essex, England) were coated with the Sephadex-gel by means of a thin-layer

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spreader (DESAGA, Heidelberg, Germany), the thickness of the layer being 0.1 cm. The coated plates were left at room temperature for 30 min immediately prior to use.

Sample solutions (5–30 μl) were applied to small pieces of Whatman No. 1 paper (up to 1 cm^2) or cover slips (1.5 X 1.5 cm) which were then placed on the thin-layer. The apparatus for IEF was similar to the one described by Awdeh et al. [6], with the exception that the two cylindrical carbon electrodes were each resting on the gel layer, the anode being in most cases close to the samples. The distance between electrodes was about 17 cm. Along anode and cathode a 5% solution of phosphoric acid or ethylene diamine, respectively, was pipetted. Focusing was carried out for 18 h at 4°C. Initially, the voltage was adjusted to 150 V; 15 h later, the power supply was turned up to 500 V. The power never exceeded 1 W.

After completion of a run, the pH gradient was measured in the middle of the Sephadex-layer with a flat membrane electrode (Dr Ingold, Zürich, Switzerland). Then the Sephadex-gel was polymerised by spraying about 3 ml of a polymerization initiating solution (1 M phosphate adjusted to pH 6.15 with Tris, 1% (v/v) *N,N,N′,N′*-tetramethylene diamine (TMED), 10% (w/v) ammonium persulphate) onto the gel followed by incubation of the plate in a nitrogen atmosphere. The polymerization was complete in 2 min when the spray-solution was not older than one week. For polymerization under milder conditions, we recommend use of the following spray: 1.6 mg riboflavin and 0.4 ml TMED dissolved in 100 ml H_2O or buffer; the plate is kept in nitrogen under u.v. light for 30 min.

Staining was carried out for 1.5 h in 0.2% bromphenolblue in ethanol–water–acetic acid (100:85:15); destaining was achieved by treatment for 6 h with ethanol–water–acetic acid (6:13:1).

Overlaying of polymerized gels with 10 μCi per plate of ^{125}I-labelled streptococcal A variant carbohydrate, subsequent processing and autoradiography were done essentially as described by Cramer and Braun [7].

3. Results and discussion

An impression of the excellent resolution of molecules in polymerizable Sephadex thin-layers can

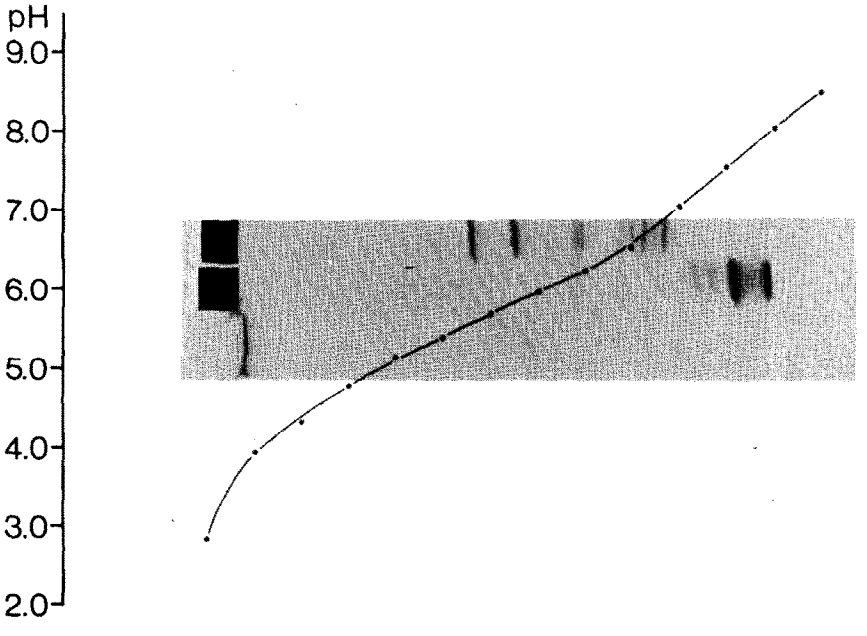

Fig.1. The isoelectric focusing patterns of 200 μg Bence–Jones protein Whi (top), 100 μg sperm whale myoglobin (middle), and 50 μg TBSV are depicted. The gel contained 2.8% (w/v) 'Ampholine' of pH range 3.5–10. To facilitate the estimation of isoelectric points of individual bands, the pH-gradient is superimposed on the photograph.

be obtained from fig.1, which shows the isoelectric focusing patterns of Bence-Jones protein Whi, sperm whale myoglobin and TBSV. The patterns and pIs of the two low molecular weight proteins correspond very well to those seen when these proteins are run in polyacrylamide gel slabs (not shown). For TBSV, a comparison could not be made, since the virions have a particle weight of $9 \cdot 10^6$ daltons [8] and are therefore excluded from polyacrylamide gels even when the concentrations of acrylamide and cross-linker are very low [1]. The isoelectric points of TBSV and of the components of sperm whale myoglobin in this experiment are in very good agreement with the values already reported [9,10]; in the case of TBSV, the pI was previously calculated from a measurement at an ionic strength of 0.02). This is so whether the samples are applied near anode or cathode (not shown). We conclude that there is no steric hindrance exerted on very large molecular aggregates like viruses and that the presence of monomeric acrylamide and methylene-bisacrylamide during the isoelectric focusing process does not interfere with the creation of a stable pH gradient and the migration of substances to positions corresponding to their correct pIs in this gradient. techniques requires that the focused proteins do not lose their binding or enzymatic activity during the polymerization of the Sephadex-layer. Fig.2 shows that binding sites of antibodies are not destroyed by the polymerization procedure since the streptococcal A variant-polysaccharide binding antibodies in two hyperimmune rabbit antisera bound the ^{125}I-labelled polysaccharide in the usual fashion. Patterns as well as pIs of these antigen-binding antibodies are very similar when experiments in polymerizable Sephadex thin-layers and polyacrylamide gel slabs are compared (D. G. Braun, unpublished results).

It has been shown [11] that focused antibodies can be crosslinked with glutaraldehyde after precipitation (18% Na_2SO_4) in order to prevent diffusion and thus loss of resolution during the time the overlay is made. IEF in polymerizable Sephadex thin-layers offers a very simple way to achieve the same effect: only the concentrations of acrylamide and cross-linking agent have to be elevated. Diffusion of focused antibody

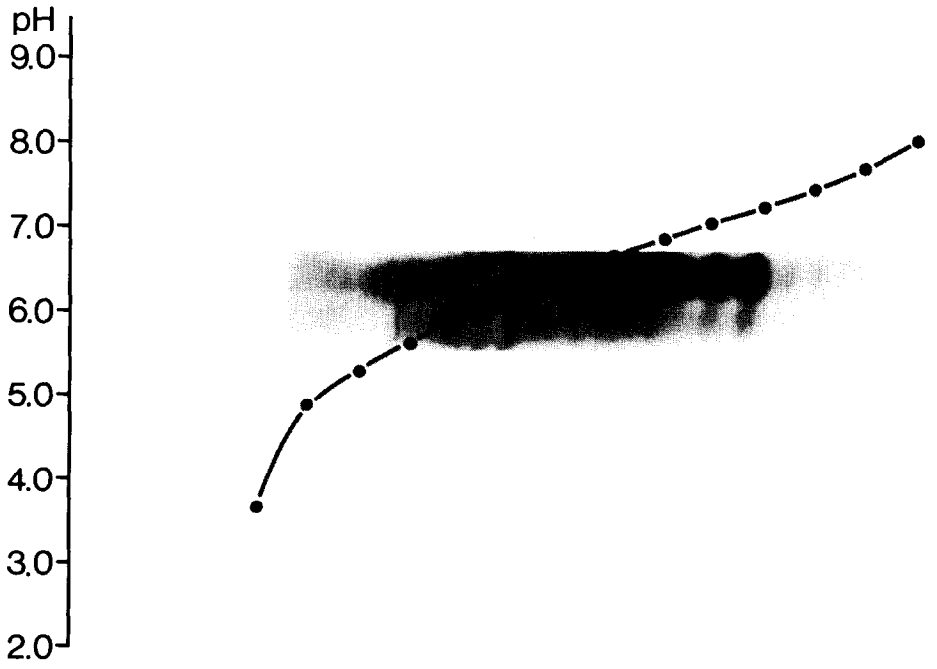

Fig.2. Autoradiography of two hyperimmune rabbit antisera (5 λ each) (6–141, top and 27–306, bottom) focused on a gel containing 2.1% pH 5–8 and 0.7% pH 7–9 'Ampholine' and overlayed with ^{125}I-streptococcal A variant polysaccharide. The film was exposed for 60 h. The pH gradient is superimposed on the photograph.

molecules can thus be prevented; very sharp banding patterns are obtainable (not shown) without the use of an additional treatment with a cross-linking agent such as glutaraldehyde.

So far, we have not carried out experiments with enzymes and we do not know whether enzymatic activity is retained in focused proteins after polymerization. If this should not be the case, it might be advantageous to polymerize under milder conditions (see Materials and methods). For a histochemical detection of enzymes, the buffer for the spray-solution can also be appropriately chosen; it is even conceivable, that the substrate(s) of enzymes could already be included in the solution initiating the polymerization.

Finally, we would like to point out that various dissociating agents, such as urea and nonionic detergents, can be mixed in high concentrations with the Sephadex gel without affecting focusing and polymerization processes (Ziegler and Köhler, unpublished results). We believe that isoelectric focusing in polymerizable Sephadex thin-layers is especially applicable for the characterization of large protein-complexes or membrane-derived glycoprotein–lipid aggregates; even large nucleic acids might be separable by this method.

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